Osteopontin Gene is Expressed in the Dermal Papilla of Pelage Follicles in a Hair-Cycle-Dependent Manner

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Hair follicle formation and maintenance involve intimate interactions between follicular epithelial cells and a group of specialized mesenchymal cells known as the dermal papilla. Using the random primer polymerase chain reaction, we have identified an ≈1.4 kb osteopontin mRNA that is present in large quantities in cultured rat vibrissa dermal papilla cells but undetectable in cultured rat skin fibroblasts. *In situ* hybridization showed that the osteopontin gene is expressed in dermal papilla cells of pelage follicles during catagen but not in anagen or telogen. As an acidic glycosylated RGD-containing extracellular matrix protein, osteopontin can function both as a cell attachment protein and as a soluble cytokine playing roles in signaling, cell migration, tissue survival, anti-inflammation, and T-cell-mediated cellular immunity. Our results indicate that the comparison of the mRNA of cultured dermal papilla cells and fibroblasts can lead to the identification of not only anagen-specific genes (e.g., nexin 1), but also a catagen-specific gene. We have thus provided evidence that specific genes are turned on during catagen, which is therefore not simply a passive "degenerative" phase. The functional role of osteopontin in catagen is unclear but it may promote the formation of a tightly aggregated dermal papilla, and/or protect the dermal papilla cells from apoptosis induced by cytokines or hypoxia during catagen.
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The interactions between dermal papilla, a group of hair-follicle-specific mesenchymal cells, and follicular epithelial cells play an important role in the regulation of hair follicle development and cyclic growth (Stenn and Paus, 2001). The specificity of the mesenchymal signal has been well established by early hair reconstitution studies showing that dermal papilla, but not skin fibroblasts, can rescue follicles devoid of the lower one third of the follicle (Oliver, 1970; Jahoda and Oliver, 1984). The diameter and length of the hair fiber are directly proportional to the volume and cell number of the dermal papilla (Van Scott and Ekel, 1958; Elliott *et al*, 1999). The androgen receptor and 5-α reductase levels of dermal papilla cells are variable and seem to play a role in androgen-dependent hair growth and androgenic alopecia (Randall *et al*, 1993, 1996; Thornton *et al*, 1996, 2001; Obana *et al*, 1997; Eicheler *et al*, 1998; Hibberts *et al*, 1998). Thus dermal papilla cells clearly play a key role in a number of important biologic processes including cell differentiation, apoptosis, hormonal regulation of cell growth, and mesenchymal–epithelial interaction.

Cells of the dermal papilla undergo striking morphologic changes as the hair follicle progresses though different phases of the hair cycle (Couchman, 1986; Messenger *et al*, 1991; Lavker *et al*, 1999). Thus, dermal papilla cells of the hair follicles that are in the growing phase (anagen) of the hair cycle are largely enclosed by follicular matrix keratinocytes. At this stage, dermal papilla cells are fibroblastic in shape, are widely interspersed by large amounts of extracellular matrix, and are supported by blood vessels. When the follicle enters into the degenerative (catagen) phase of the hair cycle, the extracellular matrix of the dermal papilla is markedly diminished, and the cells become rounded and tightly packed with greatly reduced intercellular space. In such catagen follicles, the dermal papilla is encased by a fibrous connective tissue sheath that undergoes contraction resulting in the retraction and upward movement of the condensed dermal papilla, which eventually abuts the lower end of the permanent portion of the resting (telogen) hair follicle (Breathnach and Smith, 1968; Sugiyama *et al*, 1976; Muller-Rover *et al*, 2001).

To better understand how the dermal papilla cells function in regulating the hair cycle and in mediating hair diseases such as androgenic alopecia, we sought to identify genes that distinguish dermal papilla cells from their closely related skin fibroblasts. Using the arbitrarily primed polymerase chain reaction (PCR) approach (Liang and Pardee, 1992; 1997), we have previously shown that nexin-1, a potent serine protease inhibitor, is abundantly expressed in cultured rat vibrissa dermal papilla cells, but not in similarly cultured skin fibroblasts (Yu *et al*, 1995; Sonoda *et al*, 1999). *In situ* hybridization showed that nexin-1 is expressed by dermal papilla of rat and human pelage hair follicles during anagen, but not during catagen or telogen (Yu *et al*, 1995; Jensen *et al*, 2000). In this paper, we describe the identification of another molecule, osteopontin (OPN), whose mRNA is accumulated in cultured rat vibrissa

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Abbreviation: OPN, osteopontin.

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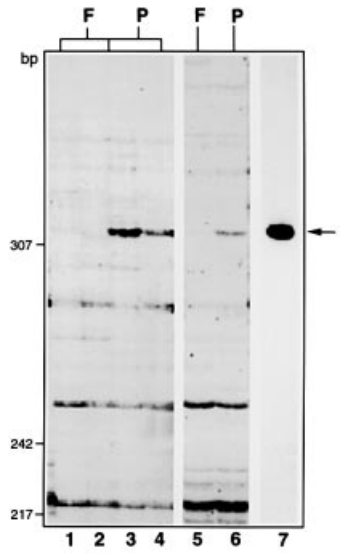

Figure 1. Identification and amplification of a dermal-papilla-specific cDNA. Total RNA (0.2 μg) isolated from cultured rat vibrissa papillary (p) cells and skin fibroblasts (F) were mixed with a specific 3′-oligo-(dT) primer, reverse transcribed, mixed with a 5′-"arbitrary" primer, and amplified by PCR. The resultant partial cDNAs were resolved electrophoretically on a DNA-sequencing polyacrylamide gel, and the partial cDNA patterns of two independent batches of cultured fibroblasts (*lanes 1*, *2*) and dermal papilla cells (*lanes 3*, *4*) generated using a particular pair of primers are shown here. Note that the two cell types shared most cDNA species, except a prominent band of 315 bp unique to the dermal papilla cells. *Lanes 5* and *6* show the patterns of fibroblasts and dermal papilla cells, respectively, in another independent experiment. This 315 bp band was excised and used as a template to generate a re-amplification product (marked by *arrow* in *lane* 7).

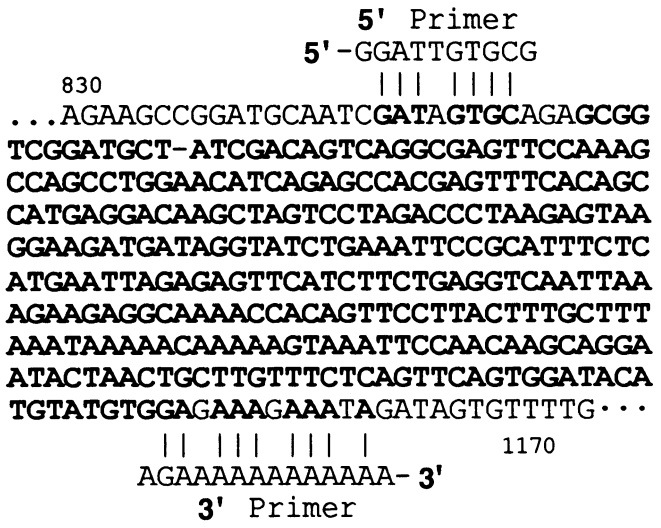

Figure 2. The 315 bp partial cDNA encodes OPN. The deoxynucleotide sequence of the 315 bp cDNA clone is shown in bold, corresponding to bases 847–1162 of a published rat OPN cDNA sequence (Giachelli *et al*, 1993). The 5′- and 3′-oligodeoxynucleotide primers used for reverse transcription PCR are indicated, with vertical lines showing identities between the primers and the OPN sequence.

dermal papilla cells but not in cultured skin fibroblasts. Interestingly, unlike nexin-1, which is limited to anagen follicles, OPN is expressed only during catagen. These results indicate that our approach of comparing cultured dermal papilla cells and skin fibroblasts allows for the identification of hair-cycle-dependent genes, and that OPN plays a role during the catagen phase of the hair cycle.

MATERIALS AND METHODS

Cell culture Vibrissa follicles were dissected individually from the lip region of (4–6-mo-old) Wistar rats, and their papillae were squeezed out using a pair of forceps. The isolated papillae were placed in a small amount of Chang's medium (1.5 ml per 60 mm) and left undisturbed in a 37°C incubator (5% CO_2) for 4 d. Under these conditions most of the papillae formed outgrowths (Jahoda and Oliver, 1981; Warren *et al*, 1992). Ten to 12 d later the cells were treated with 0.1% trypsin and 0.05% ethylenediamine tetraacetic acid in phosphate-buffered saline, and the dissociated single cells were then plated in Dulbecco's minimal essential medium containing 10% fetal bovine serum. The lip skin tissues, from which the vibrissae had been removed, were then minced thoroughly to less than 1 mm^3 and placed under outgrowth conditions and subcultured as described above. When grown under these conditions, the papillary cells and skin fibroblasts maintained distinct morphology (Jahoda and Oliver, 1984). The RNA of the cultured cells (three to four passages) were extracted in 4 M guanidinium thiocyanate, purified by ultracentrifugation, and treated with RNase-free bovine pancreatic DNase (Chomczynski and Sacchi, 1987).

Differential display of mRNA Total RNA of cultured cells were reverse transcribed and subjected to PCR using 20 3′-oligo$(dT)_{12}$ primers (14 bp) coupled with 20 5′-random primers (10 bp oligodeoxynucleotides) (Liang and Pardee, 1992). After the PCR, the cDNAs were resolved on a 6% polyacrylamide DNA sequencing gel. The area of a dried gel containing a cDNA fragment of interest was excised and extracted by heating in distilled water at 90°C for 5 min.

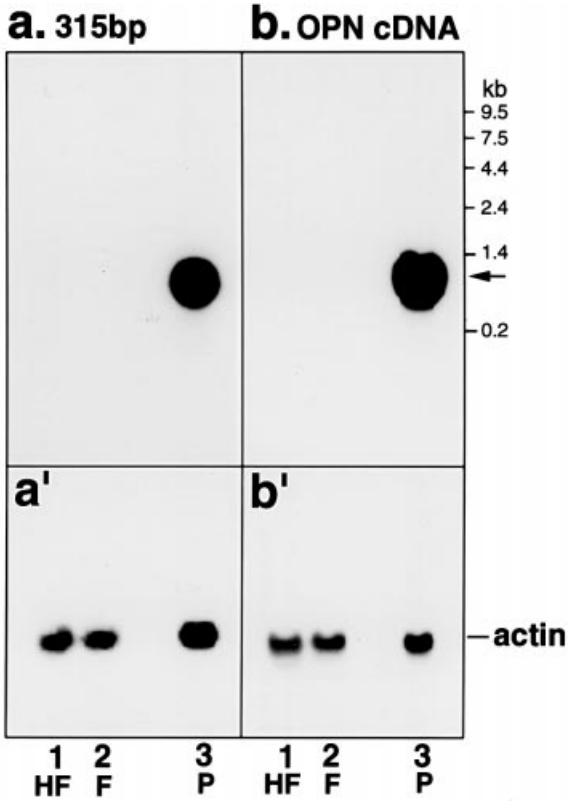

Figure 3. Follicular specificity of the OPN message as shown by northern blotting. Total RNA (20 μg) of cultured human embryonic lung fibroblasts (*lane 1*; HF), rat skin fibroblasts (*lane 2*; F), and rat vibrissa papillary cells (*lane 3*; p) were resolved electrophoretically on a formaldehyde-agarose gel, and probed with a ^{32}P-labeled 315 bp partial OPN cDNA (*a*), a full-length 1.4 kb OPN cDNA (*b*), or actin cDNA (*a′*, *b′*). Numbers on the right of panel (*b*) denote the size markers in kilobases (kb). Note that although the three cell types contained about equal amounts of actin message, a prominent ≈1.4 kb OPN mRNA (*arrow*) was detected as a major component only in the follicular dermal papilla cells.

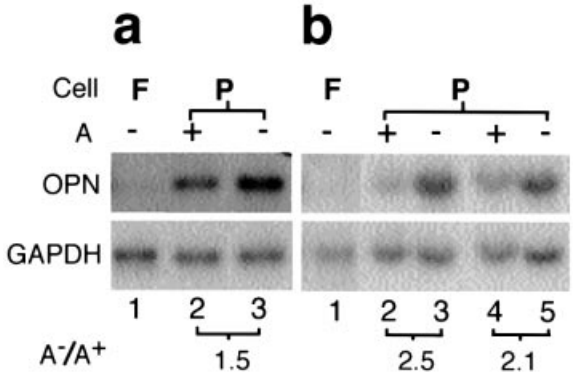

Figure 4. Inhibition of OPN expression in cultured rat vibrissa dermal papilla (p) cells by R1881, an androgen analog. Rat vibrissa dermal papilla cells (p) and fibroblasts (F) were grown in Dulbecco's minimum essential medium containing 10% fetal bovine serum. Half of the dishes were fed with the same medium containing 10 nM R1881 (+), and the other half were maintained in a regular medium (–) for 3 d postconfluence. Total RNA were extracted 3 d later from these cells and the amounts of OPN and glyceraldehyde-3-phosphate dehydrogenase were quantified by (*a*)virtual northern analyses or by (*b*) regular northern analyses. The numbers (A–/A+) indicate the ratio of OPN in regular and R1881-containing media. Panel (*b*) shows the results of two independent batches of vibrissa cells. Note the suppression of OPN expression in cultured dermal papilla cells.

The solubilized partial cDNA was used as the template for two rounds of re-amplification using the original pair of primers. After the size of the re-amplified cDNA fragment was confirmed by gel electrophoresis, the fragment was cloned into a PCRII vector and sequenced using the dideoxynucleotide procedure of Singer (US Biochemical Kit). Northern blot analysis and *in situ* hybridization were performed according to previously described procedures (Yu *et al*, 1995; Jensen *et al*, 2000).

Virtual northern analyses The cDNAs of cultured rat vibrissa dermal papilla cells and fibroblasts were synthesized using a SMART PCR cDNA synthesis kit (Clontech, Palo Alto, CA). The amplification of such cDNAs by PCR was done for 17 cycles, whereas the increase in the amounts of two major mRNA species (glyceraldehyde-3-phosphate dehydrogenase and tubulin) was still in a linear range. The same amounts of total DNA from each sample were then analyzed by southern blot.

RESULTS AND DISCUSSION

Using 20 5′-random PCR primers coupled with the same number of 3′-primers, we performed ≈400 PCR using the cDNAs of cultured rat vibrissa dermal papilla cells and skin fibroblasts as the templates. Electrophoretic comparison of the cDNAs revealed a partial cDNA of 315 bp that was abundant in cultured dermal papilla cells but virtually undetectable in cultured fibroblasts (**Fig 1**). This partial cDNA encoded OPN (**Fig 2**), a widely distributed extracellular matrix protein that can be both glycosylated and phosphorylated (see below). Northern blot analysis using this 315 bp partial cDNA and a 1.4 kb full-length OPN cDNA confirmed the marked overexpression of this gene in cultured

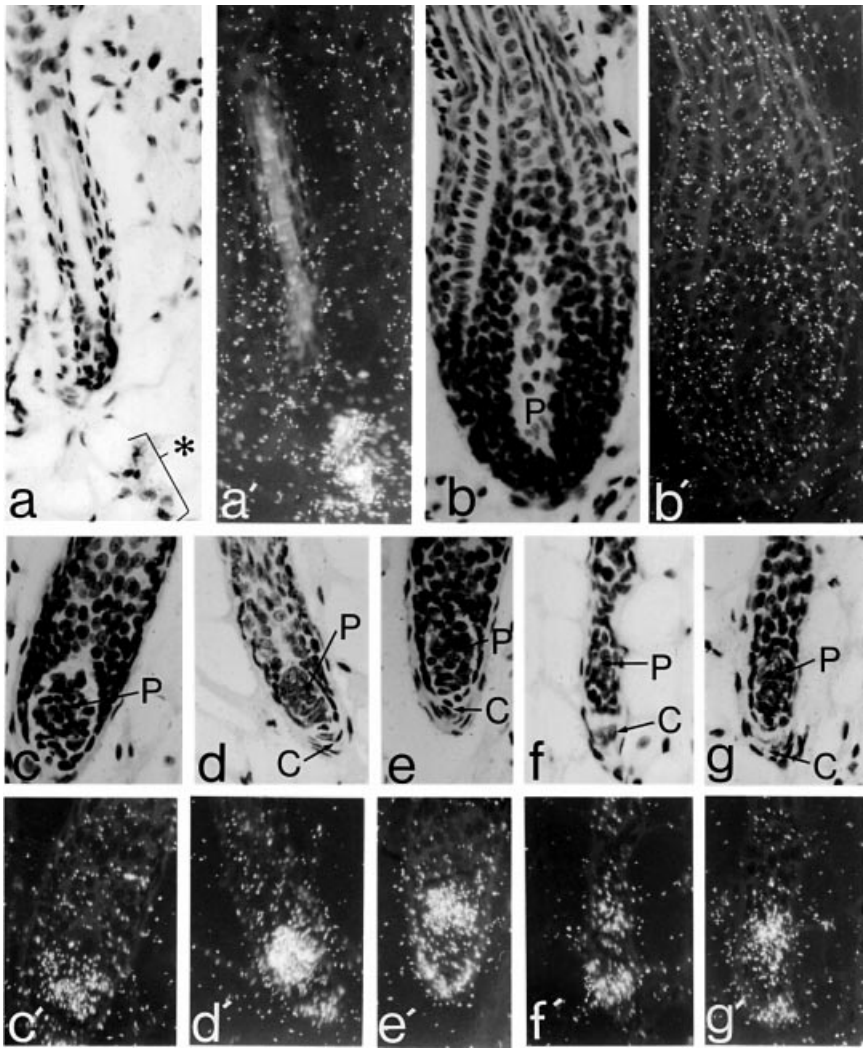

Figure 5. Expression of OPN in dermal papillae of rat catagen hair follicles. Specimens of skin were taken from rats that were (*a*) 22 d old showing a telogen follicle – note the strong OPN signals in macrophages (*asterisk*) and the lack of signals in the follicle; (*b*) 33 d old showing an anagen hair follicle – note the absence of OPN signals; (*c*) 46 d old showing a catagen-III follicle – note strong OPN signals in the partially condensed dermal papilla; (*d*, *e*) 46 d old showing two catagen-IV follicles – note strong OPN signals in the condensed dermal papilla and the newly formed trailing connective tissue sheath (labeled C); (*f*) 45 d old catagen VII – note strong OPN signals in dermal papilla and trailing connective sheath; and (*g*) 48 d old catagen III. (*a*)–(*g*) Hematoxylin and eosin staining; (*a*′)-(*g*′) dark field pictures of the corresponding fields highlighting the silver grain signals of OPN. Abbreviations: p, dermal papilla cells; C, trailing connective tissue sheath; *, macrophage. The phases of hair cycle were designated according to Muller-Rover *et al* (2001). Note the accumulation of OPN mRNA in macrophages as well as in the dermal papillary and trailing connective tissue sheath cells of the catagen follicles, and the lack of OPN expression in anagen follicles. *Scale bar*: 100 μm.

dermal papilla cells (*versus* cultured skin fibroblasts; **Fig 3**). Consistent with a previous report that androgen can suppress OPN expression in medullary carcinoma (Nagoshi *et al*, 1994), we found that the expression of OPN in cultured rat vibrissa dermal papilla cells could also be suppressed by R1881, an androgen analog (**Fig 4**).

We then analyzed the expression of OPN during different phases of the hair cycle using *in situ* hybridization (**Fig 5**). OPN mRNA was accumulated in the dermal papilla and the ''trailing connective sheath'' of a subpopulation of rat pelage follicles that were in the catagen phase of the hair cycle (**Fig 5*c–g***, ***c′–g′***). No OPN was detected in the dermal papilla of the anagen (**Fig 5*b***, ***b′***) or telogen follicles (**Fig 5*a***). Macrophages scattered around the pelage follicles contained, as expected, abundant OPN messages (**Fig 5*a***, ***a′***) (Hirota *et al*, 1993; O'Brien *et al*, 1994). Very little OPN mRNA was detected, however, in the nonfollicular dermal fibroblasts (**Fig 5**), thus confirming the dermal papilla ''specificity'' of the OPN gene.

Our approach of comparing the gene expression patterns of cultured dermal papilla cells and fibroblasts has two potential limitations. First, as cultured dermal papilla cells may mimic more closely the presumably more metabolically active cells of the anagen follicle, this approach may be limited to uncovering only those genes, such as nexin-1, that are expressed during anagen (Yu *et al*, 1995). Second, as vibrissae differ from the pelage hairs in many of their biologic features (Jahoda, 1992), the vibrissa genes we identified this way may not be expressed in the pelage hair. Our results indicate, however, that our approach can lead to the identification of dermal-papilla-related genes that are expressed during anagen as well as catagen phases of the pelage follicle.

OPN is a secreted protein with a predicted protein core molecular weight of 52 kDa that can be both glycosylated and phosphorylated (Denhardt *et al*, 2001a, b). The protein is frequently overexpressed in transformed cells (Denhardt and Guo, 1993; Rodan, 1995). Its expression is relatively tissue-specific and can be regulated by a number of hormones, growth factors, tumor promoters, and oncogenes. As the protein has an RGD domain, many cell types, including osteoblasts, osteoclasts, and transformed fibroblasts, can bind OPN via the $\alpha v\beta 3$ integrin receptor. An interesting feature is that thrombin can cleave OPN close to the RGD sequence, thus exposing the RGD site and enhancing cellular binding (Senger *et al*, 1994). Another highly unusual feature of OPN is that osteoclasts can bind only to phosphorylated OPN, and that the cells can secrete a phosphatase to self-regulate their interactions with the matrix (Ek-Rylander *et al*, 1994). Thus OPN plays diverse roles in mediating cell–matrix interaction, growth regulation, and various aspects of the inflammation/tissue repair cascade (Denhardt and Guo, 1993; Rodan, 1995; Denhardt *et al*, 2001a, b). Our study provides the first evidence that OPN may also play a role in hair physiology.

The mechanism for the upregulation of the OPN gene in dermal papilla of catagen follicles is unclear. Either the gene is activated intrinsically in response to a set of catagen-specific transcription factors, or it is secondarily induced by extracellular signals including cytokines (Denhardt and Guo, 1993; Rodan, 1995), extracellular matrix changes (Carvalho *et al*, 1998), or oxidants (Ramos, 1999) that are known to induce the OPN gene.

Although the functional role of OPN in hair regulation is not yet known, it may be involved in three aspects of hair growth. As indicated earlier, we have previously demonstrated that the dermal papilla cells of growing follicles express a high level of nexin-1 mRNA, which encodes a potent inhibitor of serine proteases including thrombin (Yu *et al*, 1995; Sonoda *et al*, 1999; Jensen *et al*, 2000). The cessation of nexin-1 expression at the end of the growing phase may allow protease activation of OPN, thus enhancing the attachment of dermal papilla cells to OPN and facilitating the aggregation of dermal papilla. Another possible function of OPN in hair regulation relates to its ability to inhibit inflammatory mediator-induced expression of the NO synthetase gene and the production of nitric oxide (Rollo *et al*, 1996; Scott *et al*, 1998). This may promote the survival of cells exposed to hypoxia (Denhardt *et al*, 1995; Denhardt and Noda, 1998). Such a protective effect of OPN suggests several potential roles. For example, OPN may protect dermal papilla cells from the cytotoxic effects of nitric oxide, which can be produced by macrophages (**Fig 4*a***, ***a′***), during catagen at a time of widespread cell destruction and deprived blood supply (Dippel *et al*, 1994; Feng *et al*, 1995). The putative protective function of OPN provides a possible mechanism of androgenic alopecia (Schilli *et al*, 1998). We showed that androgen can suppress OPN expression in cultured rat vibrissa dermal papilla cells (**Fig 4**; Nagoshi *et al*, 1994). If such androgen suppression of OPN expression also occurs in human scalp follicles, this could compromise the protective function, thus resulting in a loss of dermal papilla cells. Yet another recently described function of OPN has to do with cellular immunity. The sensitization phase of experimentally induced contact hypersensitivity is hair cycle dependent (i.e., sensitization during anagen fails to elicit ear swelling whereas sensitization during telogen results in marked ear swelling). This observation suggests a hair-cycle-dependent mechanism whereby only an anagen follicle can produce immunosuppressive factors thus leading to a temporary state of unresponsiveness to a hapten, i.e. the follicle becomes immune privileged (Paus *et al*, 1999, and references therein). OPN has recently been shown to upregulate interleukin-12 and suppress interleukin-10 activity, making it an early component of type-1 immunity (Ashkar *et al*, 2000). Thus, OPN secreted by dermal papilla during catagen may be able to trigger a type 1 immune response mediated by effector cytokines such as interferon-γ in conjunction with a dampening of antiapoptotic factors (e.g., interleukin-4), resulting in the destruction of the lower portion of the follicle. Additional studies are needed to test these several possibilities.

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